

AWARD NUMBER: W81XWH-16-1-0574

TITLE: Apyrase: A Portable Treatment to Prevent Burn Progression and Infection

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REPORT DATE: September 2017

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE

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1. REPORT DATE September 2017			2. REPORT TYPE Annual		3. DATES COVERED 1 Sep 2016 - 31 Aug 2017	
4. TITLE AND SUBTITLE Apyprase: A Portable Treatment to Prevent Burn Progression and Infection					5a. CONTRACT NUMBER	
					5b. GRANT NUMBER W81XWH-16-1-0574	
					5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Stewart C. Wang, MD, PhD Jean Nemzek, PhD Benjamin Levi, MD Chuanwu Xi, PhD					5d. PROJECT NUMBER	
					5e. TASK NUMBER	
					5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Regents of the University of Michigan Kathryn DeWitt 503 Thompson St. Ann Arbor, MI 48109-1340					8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012					10. SPONSOR/MONITOR'S ACRONYM(S)	
					11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited						
13. SUPPLEMENTARY NOTES						
14. ABSTRACT Year one focused on administrative and regulatory requirements, initiation of experimentation, assessment of techniques, and analysis of preliminary results. This laid the ground work for subsequent experimentation. IACUC and ACURO approvals were obtained in the first quarter, along with approval for Specific Aim 2 bacterial studies. In vivo experimentation began in the third quarter and was continued in the fourth quarter. Methods for anesthesia, analgesia and bandaging were confirmed and standardized. The parameters for burn wounds (duration of contact and placement/spacing). The randomization and the delivery method for the test agents were determined. Sample collection strategies were confirmed. Sample collection, processing and analysis began in the fourth quarter. This includes processing of the histology samples and swabs for ATP assays taken at each time point for each animal. Photographs of each wound over the course of the experiment have been archived. Our goal was to have all of the animal experimentation for the first specific aim completed in the first year. This was not realized. However, we have optimized our study design and methods and are confident that progress be rapid and the project will be completed on time. Preliminary data suggest that apyprase reduces the size of partial thickness burns within the first three days.						
15. SUBJECT TERMS Inflammation, Thermal, Healing, Antimicrobial, Enzyme, Infection, Biofilm						
16. SECURITY CLASSIFICATION OF: U			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC	
a. REPORT Unclassified	b. ABSTRACT Unclassified	c. THIS PAGE Unclassified	Unclassified	12	19b. TELEPHONE NUMBER (include area code)	

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1. INTRODUCTION:

In the management of thermal injury, the major goals of initial, non-surgical treatment are reduction of local inflammation, prevention of wound progression, and inhibition of bacterial infection. However, at this time, there are no effective treatments to reduce wound progression and the emergence of resistant bacteria has threatened the efficacy of antibiotic therapy. In this study, we propose that apyrase, an ATP hydrolytic enzyme, would fulfill the unmet need for an effective topical treatment for burn injury. Excessive extracellular ATP (eATP) released from injured tissues acts as a danger-associated molecular pattern, triggering inflammatory responses, and eATP also promotes biofilm formation in several strains of bacteria. Apyrase hydrolyzes ATP to ADP and phosphate which has effectively controlled the inflammatory response in our previous work in mouse models of thermal injury and associated complications. This current study will further examine the use of apyrase in a relevant porcine model. The study is designed to quantify healing, inflammation, and bacterial infection of burn wounds to compare the efficacy of apyrase with that of controls and standard of care topical therapy. The application of apyrase will be tested in partial thickness burns and repeated in burns with concurrent bacterial contamination. Outcome measures to be assessed include gross wound characteristics, histology, inflammation, and biofilm content. These measures will be used to assess the known anti-microbial, anti-inflammatory, and pro-healing effects of apyrase and act as a step towards translation of this treatment into burn wound therapy. Ultimately the goal is to improve recovery time, reduce costs, and improve outcomes for many burn patients.

2. KEYWORDS:

Inflammation
Thermal
Healing
Antimicrobial
Enzyme
Infection
Biofilm

3. ACCOMPLISHMENTS:

- **What were the major goals of the project?**

Specific Aim 1: Demonstrate that topical apyrase decreases inflammation and wound expansion of partial thickness burns. (Months 1-12)

Subtask 1: Animal Use Approval: completed 11/14/2016

Subtask 2: Burn analysis for inflammation and progression: 20% complete

Subtask 3: Conduct design of experiments analysis using pig model: completed 8/21/2017

Specific Aim 2: Validate the antimicrobial properties of topical apyrase in partial thickness burns. (Months 12-36)

Subtask 1: Perform burn model with gram negative and gram-positive infection

Subtask 2: Burn analysis of inflammation and progression

Subtask 3: Quantify bacterial load, biofilm and data analysis

- **What was accomplished under these goals?**

- 1) major activities

The first year of this project focused on administrative and regulatory requirements, initiation of experimentation, assessment of techniques, and analysis of preliminary results. This year laid the ground work for subsequent years of experimentation.

For regulatory issues, initial IACUC and ACURO approvals were obtained in the first quarter, along with approval for Specific Aim 2 bacterial studies from the Institutional Biosafety Committee and Environmental Health Services at the university. At the end of the third quarter, the renewal to the IACUC protocol was submitted. Toward the end of the approval process, the university announced new training requirements for all animal investigators. This delayed the approval until all personnel could complete multiple training modules. The approval was obtained in the fourth quarter and we are now awaiting the ACURO approval for the renewal protocol.

Organization and preparation occurred in the first and second quarters. These activities included scheduling with vendors, animal housing, and procedure rooms as well as ordering all operative and bench supplies.

The in vivo experimentation began in the third quarter and was continued in the fourth quarter. Methods for anesthesia, analgesia and bandaging were confirmed and standardized. The parameters for burn wounds (duration of contact and placement/spacing). The randomization and the delivery method for the test agents were determined. Sample collection strategies were confirmed and are now routine procedures.

Sample processing and bench analyses began in the fourth quarter. This includes processing of the histology samples and ATP assays taken at each time point for each animal. Photographs of each wound over the course of the experiment have been archived.

2) specific objectives

Specific Aim One: Demonstrate that topical apyrase decreases inflammation and wound expansion of partial thickness burns.

Methods: Pigs (Yorkshire-cross, females, 25-30 kg) were acclimated for at least 5 days prior to studies. On the day of procedure, the pigs are sedated with telazol/xylazine and anesthesia is maintained on isoflurane via face mask. A buprenorphine patch is applied and one injection of buprenorphine is given pre-emptively. After appropriate skin reparation, eight burn wounds are created with a heated metal block (5 x 5cm, 80°C). The cranial and dorsal corners are tattooed to allow measurement over time (Figure 1). The treatments are assigned to wounds randomly and included a saline control, standard of care treatment, and two doses of apyrase (0.5 or 1.0 Units). For the initial experiments, silver sulfadiazine was used as the standard of care and applied as per manufacturer directions. However, residual silver sulfadiazine appears to interfere with our collection of samples on successive days. Therefore, we proposed using Sulfamylon (malfenic acid) as the standard of care, since it can be applied in a fashion identical to the apyrase and represents a frequently used treatment of burn wounds. This change has been submitted for ACURO approval in the new document. All treatments can be applied with a spray bottle that delivers a standardized volume. The wounds are bandaged in several layers and covered by a jacket. On Days 0, 1, 3, 7, 14, the animals are anesthetized. Each wound is photographed. Measurements are taken of the distance between the two cranial corners and the two dorsal corners of each wound. A sterile cotton swab is rolled over the entire wound surface and then placed in sterile saline. Two (3mm) biopsy punches are obtained (one central and one in a corner of the wound) and are placed in formalin. On day 21, the biopsy procedures are repeated, an additional sample is taken for RNA, and then the entire wound is resected and placed in for storage in formalin.

The objectives are to measure ATP levels, quantify inflammation, and determine effects on wound size/depth using the methods described above.

3) significant results or key outcomes

Animal well-being: All of the animals on study to date have responded well to the anesthetic and analgesic regimen outlined. None of the animals have required rescue analgesia during the study and have maintained appropriate body condition. A recent workshop on pig anesthesia suggested that oral diazepam may help reduce stress of handling. This was added to the renewal

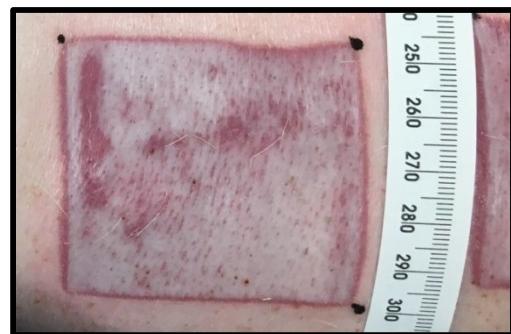


Figure 1: Initial Burn Wound. A burn created with a 5x5cm metal block heated to 80°C. Three corners have been tattooed to allow measurement of the cranial and dorsal edges of the wound.

of the animal use protocol and has been approved by the IACUC, pending ACURO approval it will be assessed in the study animals.

Wound progression: In the model, eight partial thickness burn wounds were created. Wounds were uniform. Block placement on the caudal most wounds may be challenging and requires extra attention due to the body contour of some animals. Wounds were delineated by a defined hyperemic border until Day 3. Subjectively there did not appear to be overlap of any inflammatory zone among the wounds, suggesting adequate placement to negate effects of multiple wounds. By Day 7, borders of the wounds become less well defined and hair growth is very noticeable. By Day 21, the wounds have patchy, mildly hyperemic areas with some scaling of the skin and pronounced hair growth. The degree of inflammation and hair growth appears more pronounced on some wounds compared to others; however, the photos remain blinded at this time to prevent bias until the cohort has been completed.

ATP assays: Swabs are taken of each wound at each time point prior to the biopsy. Samples from the first experimental animal tested below the limit of detection for the majority of samples. The sampling procedure was then changed so that after collection, the swabs would be placed in a smaller amount of liquid to elute the contents at a greater concentration. Subsequent samples tested at this higher concentration. Therefore, these data are pending.

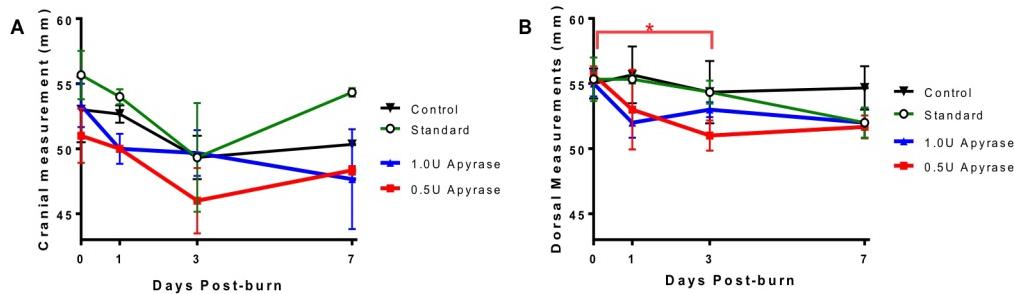


Figure 2. Wound measurements. The distance between tattoo marks at the cranial and dorsal edges of the wound was measured at the time of injury and at bandage changes. Data are expressed as mean \pm SEM. * = $p < 0.05$ by repeated measures ANOVA. n=3.

Assessment of wound size: On Day 0 and each subsequent day of biopsy and bandage change, the distance between tattoo marks placed at the corners of the wound was measured. For all treatments, the cranial edge of the burn wounds decreased over the first three days (Figure 2). The standard of care treatment and the 0.5 Unit apyrase demonstrated the most dramatic changes relative to the baseline wound size, although these findings were not significant. For the measurements on the dorsal aspect of the wound, the control wounds demonstrated little change in overall size. The treatment with 0.5 U apyrase demonstrated a statistically significant decrease in size, while the higher dose (1.0 U) showed similar not statistically significant trends. These preliminary data suggest that the apyrase treatment may decrease

wound size within the first three days. Further work is needed to determine whether this difference is biologically significant.

Histology: Biopsy samples were taken on Days 0, 1, 3, 7, 14, and 21 from the center of the wound and from the four corners of the wounds. Biopsy sites were spaced in order to decrease potential effects from serial sampling. Standardized, serial full thickness biopsies will be evaluated for indicators of inflammation (neutrophil counts, proinflammatory cytokine expression) and wound progression (necrosis, epithelialization, Ki-67 and apoptosis-TUNEL immunostains). The optimal dose of apyrase will be defined by comparisons between treatments for reduction of inflammation and necrosis. Routine histology has been processed. Histomorphometric and special staining studies are pending at this time. The results will provide critical information on the kinetics of healing with and without apyrase.

4) goals not met

Our goal was to have all of the animal experimentation for the first specific aim completed in the first year. This was not realized. However, we have optimized our study design and methods and are confident that progress be rapid and the project will be completed on time.

- **What opportunities for training and professional development has the project provided?**
Nothing to report
- **How were the results disseminated to communities of interest?**
Nothing to report
- **What do you plan to do during the next reporting period to accomplish the goals?**

By the next annual report, we will have all of specific aim one and half of specific aim two completed. This will include finishing our initial animal experiments to determine the dose of apyrase to use on subsequent studies. We will also perform the studies of burn wounds infected with gram negative bacteria and begin the assays for the presence of biofilm.

4. **IMPACT:**

- **What was the impact on the development of the principal discipline(s) of the project?**
Nothing to report
- **What was the impact on other disciplines?**
Nothing to report
- **What was the impact on technology transfer?**
Nothing to report
- **What was the impact on society beyond science and technology?**
Nothing to report

5. CHANGES/PROBLEMS:

- **Changes in approach and reasons for change**
Nothing to report
- **Actual or anticipated problems or delays and actions or plans to resolve them**
Minor delays were experienced over the past year with regard to administrative and regulatory requirements at the institutional level. However, these have been rectified and no further delays are expected.
- **Changes that had a significant impact on expenditures**
Nothing to report
- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
- **Significant changes in use or care of human subjects**
Not applicable
- **Significant changes in use or care of vertebrate animals.**
There have been no major changes to the care and use of animals on this study. Minor changes were made to allow better sedation and less stress to the animals. These are pending approval by ACURO.
- **Significant changes in use of biohazards and/or select agents**
Nothing to report

6. PRODUCTS:

- **Publications, conference papers, and presentations**
 - **Journal publications.**
Nothing to report
 - **Books or other non-periodical, one-time publications.**
Nothing to report
 - **Other publications, conference papers, and presentations.**
Nothing to report
- **Website(s) or other Internet site(s)**
Nothing to report
- **Technologies or techniques**
Nothing to report
- **Inventions, patent applications, and/or licenses**
Nothing to report
- **Other Products**
Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- o What individuals have worked on the project?

Name:	Stewart C. Wang, MD, PhD
Project Role:	PI
Nearest person month worked:	1
Contribution to project:	Dr. Wang is the PI on this grant with expertise in acute burn care
Funding Support Change:	No change

Name:	Jean Nemzek, PhD
Project Role:	Co-Investigator
Nearest person month worked:	1
Contribution to project:	Dr. Nemzek has extensive large animal surgical experience and expertise managing animal surgical experiments and projects.
Funding Support Change:	New NIH grant R01 Takayama, Shuichi role: Co-PI

Name:	Benjamin Levi, MD
Project Role:	Co-Investigator
Nearest person month worked:	1
Contribution to project:	Dr. Levi has acute burn care expertise
Funding Support Change:	No change

Name:	Chuanwu Xi, PhD
Project Role:	Co-Investigator
Nearest person month worked:	1
Contribution to project:	Dr. Xi's lab specializes in biofilms
Funding Support Change:	No change

- o Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
- o What other organizations were involved as partners?
Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

- o COLLABORATIVE AWARDS:

Nothing to report

- o QUAD CHARTS:

See Appendix A

9. APPENDICES:

Appendix A: Quad Chart

Appendix A: Quad Chart

Apyrase: A Portable Treatment to Prevent Burn Progression and Infection

MB150237

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PI: Wang, Stewart C.

Org: University of Michigan

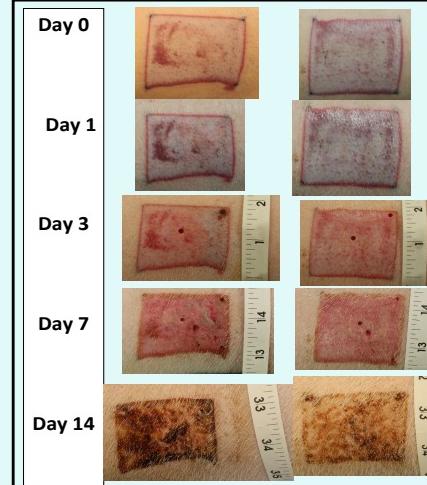
Award Amount: \$750, 000

Study/Product Aim(s)

- Specific Aim I: Demonstrate that apyrase decreases inflammation and wound expansion of partial thickness burns without toxicity.
- Specific Aim II: Validate the anti-microbial properties of apyrase in partial-thickness burns.

Approach

Apyrase will be tested in a porcine model of multiple partial thickness burns produced by standardized thermal contact. First, an optimal dose will be determined by comparing inflammation and wound progression after treatment with apyrase. The optimal dose will be evaluated for evidence of local and systemic toxicity. Finally, the antimicrobial effects of apyrase will be tested in burns infected with bacteria.



Accomplishment: Porcine In vivo studies are currently underway.

Timeline and Cost

Activities	CY	16	17	18	19
Demonstrate effectiveness					
Validate anti-microbial properties					
Infection studies					
Data analysis & Preparation for possible clinical trials					
Estimated Budget (\$K)	\$250	\$250	\$250		

Goals/Milestones

CY17 Goal – Demonstrate topical apyrase decreases inflammation and wound expansion of partial thickness burns

- 100% Animal Use Approval (completed 11/16)
- 100% Design of experiment analysis using pig model
- 25% Burn analysis for inflammation progression

CY18 & Goal – Validate the anti-microbial properties of topical apyrase in partial-thickness burns

- 0% Perform burn model with gram negative infection
- 0% Burn analysis of inflammation and progression

CY19 Goal – Infection studies and future directions

- 0% Quantify bacterial load, biofilm and data analysis

Comments/Challenges/Issues/Concerns

No budget or other concerns at this time

Budget Expenditure to Date

Projected Expenditure: \$125,000

Actual Expenditure: \$83,674